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Fatal exudative dermatitis in island populations of red squirrels (*Sciurus vulgaris*): spillover of a virulent *Staphylococcus aureus* clone (ST49) from reservoir hosts

Kay Fountain^{1,*}, Tiffany Blackett², Helen Butler³, Catherine Carchedi⁴, Anna-Katarina Schilling⁵, Anna Meredith^{5,6}, Marjorie J. Gibbon¹, David H. Lloyd⁴, Anette Loeffler⁴ and Edward J. Feil¹

Abstract

Fatal exudative dermatitis (FED) is a significant cause of death of red squirrels (*Sciurus vulgaris*) on the island of Jersey in the Channel Islands where it is associated with a virulent clone of *Staphylococcus aureus*, ST49. *S. aureus* ST49 has been found in other hosts such as small mammals, pigs and humans, but the dynamics of carriage and disease of this clone, or any other lineage in red squirrels, is currently unknown. We used whole-genome sequencing to characterize 228 isolates from healthy red squirrels on Jersey, the Isle of Arran (Scotland) and Brownsea Island (England), from red squirrels showing signs of FED on Jersey and the Isle of Wight (England) and a small number of isolates from other hosts. *S. aureus* was frequently carried by red squirrels on the Isle of Arran with strains typically associated with small ruminants predominating. For the Brownsea carriage, *S. aureus* was less frequent and involved strains associated with birds, small ruminants and humans, while for the Jersey carriage *S. aureus* was rare but ST49 predominated in diseased squirrels. By combining our data with publicly available sequences, we show that the *S. aureus* carriage in red squirrels largely reflects frequent but facile acquisitions of strains carried by other hosts sharing their habitat ('spillover'), possibly including, in the case of ST188, humans. Genome-wide association analysis of the ruminant lineage ST133 revealed variants in a small number of mostly bacterial-cell-membrane-associated genes that were statistically associated with squirrel isolates from the Isle of Arran, raising the possibility of specific adaptation to red squirrels in this lineage. In contrast there is little evidence that ST49 is a common carriage isolate of red squirrels and infection from reservoir hosts such as bank voles or rats, is likely to be driving the emergence of FED in red squirrels.

DATA SUMMARY

All genomes have been deposited with GenBank under Bioproject PRJNA679188 (accession numbers are given in Table S1, available in the online version of this article). Closed genome accession numbers: CP065353.1, CP065354.1 and CP065355.1.

The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary data files.

INTRODUCTION

Red squirrels (*Sciurus vulgaris*) have been extirpated from most of the UK, remaining only in pockets of the north of England,

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Author affiliations: ¹Department of Biology and Biochemistry, University of Bath, Claverton Down, Bath, BA2 7AY, UK; ²Voluntary Co-ordinator of the JSPCA Animals' Shelter Red Squirrel Disease Surveillance Scheme, JSPCA Animals' Shelter, 89 St Saviours Road, St Helier, Jersey JE2 4GJ, Jersey; ³Wight Squirrel Project, PO Box 33 Nicholson Road, Ryde, Isle of Wight PO33 1BH, UK; ⁴Department of Clinical Science and Services, Royal Veterinary College, Hatfield, North Mymms, Hertfordshire, AL9 7TA, UK; ⁵The Royal (Dick) School of Veterinary Studies and The Roslin Institute, University of Edinburgh, Easter Bush Campus, Midlothian, Edinburgh, EH25 9RG, UK; ⁶Melbourne Veterinary School, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville Campus, Melbourne, VIC, 3010, Australia.

*Correspondence: Kay Fountain, kif21@bath.ac.uk

Keywords: fatal exudative dermatitis; red squirrels; *Staphylococcus aureus*; transmission; wildlife hosts.

Abbreviations: BLAST, Basic Local Alignment Search Tool; BRIG, BLAST ring image generator; CI, Channel Islands; FED, fatal exudative dermatitis; GWAS, genome wide association study; IEC, immune evasion complex; IOW, Isle of Wight; JSPCA, Jersey Society for Prevention of Cruelty to Animals; LukM, component of leucocidin LukM/F encoded by *lukM* gene; MLST, multi locus sequence type; SNP, single nucleotide polymorphism.

All genomes have been deposited with GenBank under Bioproject PRJNA679188. Closed genome accession numbers: CP065353.1, CP065354.1, and CP065355.1. This article contains data hosted by Microreact.

Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Four supplementary figures and five supplementary tables are available with the online version of this article.

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Scotland and on islands such as the Isle of Arran (Scotland), the Isle of Wight (IOW) (England) and Jersey in the Channel Islands where the competing non-native grey squirrel (*Sciurus carolinensis*) has not spread [1]. Red squirrels themselves were introduced to Jersey and to the Isle of Arran during the nineteenth and twentieth centuries respectively, and elsewhere in the British Isles historical population declines have been reversed by the introduction of animals from Europe and Scandinavia [1]. Red squirrels suffer from a number of skin diseases including poxvirus and leprosy, which can be fatal and threaten remaining populations [2, 3]. Recently, another fatal skin disease called 'fatal exudative dermatitis' (FED) has affected red squirrels on the IOW and Jersey, where surveillance found the disease in 15% of all examined dead squirrels [4]. Gross findings include an exudative, ulcerative, necrotic dermatitis around the mouth, nose and feet of affected squirrels, often with necrosis of digits [5]. The histopathology of affected skin shows epidermal hyperplasia and hyperkeratosis with Gram-positive cocci in surface crust and intraepidermal pustules. In some cases *S. aureus* has been isolated from the lung and liver of affected squirrels, consistent with systemic spread of infection [5]. Investigations into the cause of this disease indicated an association with a *Staphylococcus aureus* multilocus sequence type (MLST) ST49 clone, which contained a gene encoding for the leucocidin LukM' (*lukM*) [6]. The results of this study suggested that *S. aureus* may not be part of the normal commensal flora of red squirrels, however this conclusion was based on samples from only a small number of unaffected squirrels ($n=14$).

S. aureus ST49 has previously been isolated from small mammals [7, 8], from pig noses [9], and from humans, including the well-characterized Tager 104 strain, which was isolated in 1947 [10]. When different strains of *S. aureus* isolated from wild rodents were tested in laboratory mouse models of virulence, the ST49 strain (strain DIP) was found to be by far the most virulent [11]. Strain DIP also carries *lukM*, and experiments in cattle demonstrated that strains from other lineages expressing high levels of LukM' were found to cause the most severe mastitis [12]. The bicomponent leucocidin LukM/F' has previously been found to be expressed by mainly bovine *S. aureus* isolates of multiple lineages [13], and was found to lyse bovine leucocytes more effectively than human [14]. The effect of LukM/F' on wild rodents which carry ST49 is not understood.

The aim of this study was to use whole-genome sequencing to compare isolates of *S. aureus* recovered from healthy carriage and cases of FED in discrete island red squirrel populations. This should shed light on the overall rate of *S. aureus* carriage and how this varies between populations, and the degree to which carriage and disease isolates are specifically adapted to red squirrels or reflect more transient spillover from sympatric reservoir hosts. Healthy squirrels on the Isle of Arran in Scotland, Brownsea Island in Poole Harbour (England) and on Jersey, and squirrels with FED on Jersey and the IOW were sampled. We sampled 228 red squirrels, and characterized 228 *S. aureus* isolates recovered from 26 healthy squirrels, from 16 squirrels with clinical signs of FED and one with non-FED skin lesions. The carriage rate and ST profiles varied markedly amongst the red squirrel populations, suggesting that carriage is largely driven by facile transmission ('spillover') of *S. aureus* isolates from other hosts. However, a genome-wide

Impact Statement

Populations of endangered wildlife are threatened by many pressures such as loss of habitat, hunting and climate change. However, in some cases, such as Chytrid disease in frogs, or poxvirus disease in red squirrels, disease can be a significant cause of population decline, leading to local loss of a species or even global extinction. The investigation of wildlife disease is often given a low priority unless it directly affects human wellbeing. This study is an important contribution to our understanding of a disease that affects a population of threatened small mammals, and to our knowledge of how a common bacterium is transmitted amongst farmed animals, humans and wildlife. Red squirrels on the islands of Jersey (Channel Islands) and the Isle of Wight have been affected by a fatal skin disease associated with a particular clone of the bacterium, *Staphylococcus aureus*. In this study we sought to establish where squirrels carry this clone, and other *S. aureus* strains, and how these findings correlate with the incidence of the disease. Our data confirm a strong association between the clone and disease, and suggest that ST49 is rarely carried by healthy squirrels, pointing to a high level of virulence. In contrast, other island squirrel populations where the disease is not so common tend to acquire other *S. aureus* strains from co-residing host species.

association (GWAS) analysis also provided preliminary evidence of specific adaptation to red squirrels on the Isle of Arran within the ruminant lineage ST133. Our data strongly reinforce the previously reported association of FED with *S. aureus* ST49 strains, and suggest that spillover from reservoir hosts drives the spread of this clone within populations of red squirrels [6].

METHODS

Sampling and isolation of staphylococci

A total of 228 red squirrels was sampled on the Isle of Arran, Brownsea Island, the IOW and Jersey (Channel Islands), between 2016 and 2019 as part of three projects, all approved by their relevant ethics oversight boards and licensing authorities [4, 15, 16]:

- (1) Squirrels trapped and anaesthetized for a separate leprosy study were swabbed opportunistically on the Isle of Arran (UK) ($n=44$) and Brownsea Island ($n=44$) [15].
- (2) On the IOW, three sick squirrels were sampled before treatment as part of the Wight Squirrel Project.
- (3) Sick, injured, orphaned and casualty red squirrels on Jersey ($n=137$) were sampled as part of the JSPCA squirrel surveillance project [4, 16].
- (4) For comparison purposes, on the Isle of Arran 20 domestic sheep from a flock grazing in the squirrel-trapping site, plus ten freshly culled wild red deer (*Cervus elaphus*) were also sampled.

For each squirrel, oral mucosae (where possible the oropharynx), the clean skin of the axilla or inguinal region and skin lesions were sampled using separate cotton tipped swabs. Swabs were rolled over each site for 2–5 s and placed into charcoal Amies medium (Technical Services Consultants, Heywood, UK) for transport to the laboratory.

Within 24 h of receipt, swabs were aseptically cut into tryptone soya broth (TSB) containing 10% salt (TSB+) (Oxoid, Thermo Fisher Scientific, Basingstoke, Hampshire, UK, and Sigma-Aldrich, Gillingham, Dorset, UK) and incubated at 37 °C for 24 h before streaking onto Columbia 5% sheep blood agar (Oxoid) and further incubation at 37 °C for 24 h. Presumptive *S. aureus* isolates were identified on the basis of colony morphology, haemolysis, and production of DNase (Oxoid) and up to five colonies were picked from different areas of the plate for each swab, yielding up to 20 isolates from a single animal. Isolates were stored at –20 °C and subcultures of 228 *S. aureus* isolates were dispatched on beads (Microbank, Prolab Diagnostics, Cheshire, UK) to MicrobesNG (Birmingham, UK) for sequencing.

Sequencing

Isolates ($n=228$) were sequenced on an Illumina platform by MicrobesNG (Birmingham) as follows:

Three beads were washed with extraction buffer containing lysostaphin and RNase A, and incubated for 25 min at 37 °C. Proteinase K and RNaseA were added and incubated for 5 min at 65 °C. Genomic DNA was purified using an equal volume of SPRI beads and resuspended in EB buffer. DNA was quantified in triplicates with the Quantit dsDNA HS assay in an Eppendorf AF2200 plate reader. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with the following modifications: two nanograms of DNA instead of one were used as input, and PCR elongation time was increased to 1 min from 30 s. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on the Illumina HiSeq using a 250 bp paired end protocol.

Three isolates were also sequenced using long-read sequencing using Oxford Nanopore Technologies (ONT):

Approximately 2×10^9 cells were used for high molecular weight DNA extraction using Nanobind CCB Big DNA Kit (Circulomics, Maryland, USA). DNA was quantified with the Qubit dsDNA HS assay in a Qubit 3.0 (Invitrogen, Eppendorf UK, UK). Long-read genomic DNA libraries were prepared with Oxford Nanopore SQK-RBK004 kit and/or SQK-LSK109 kit with Native Barcoding EXP-NBD104/114 (ONT, UK) using 400–500 ng of high molecular weight DNA. Twelve to twenty-four barcoded samples were pooled together into a single sequencing library and loaded in a FLO-MIN106 (R.9.4 or R.9.4.1) flow cell in a GridION (ONT, UK).

Additional sequences from seven red squirrels from Jersey and the IOW, which were part of the 2013 investigation were obtained

from public databases using metadata kindly supplied by the authors, and other sequences from CC49 were identified using pubMLST and downloaded from GenBank (Table S2) [6, 17, 18].

Bioinformatics

Quality control, assembly and annotation of reads was undertaken using an in-house pipeline as follows: raw reads were trimmed using Trimmomatic [19], and quality confirmed with Fastqc [20]. Reads were assembled using Spades [21], and annotated with Prokka [22]. Hybrid assemblies of Illumina and Nanopore reads were built using Unicycler [23], and the assembly graphs visualized using Bandage [24]. MLSTs were determined using the software tool 'MLST' (profiles in Table S3) [17, 25]. Variants were called using Snippy against closed genome assemblies from this study as references and Snippy-Core used to generate full genome alignments [26]. Snp-dists was used to produce a distance matrix from an alignment [27].

Pangenomes were built using Roary [28], and phylogenetic trees constructed from the core gene alignments using RAXML-NG [29]. Phylogenetic tree illustrations were produced in iTOL v5.7 [30]. Genomes were visually examined and manipulated using Artemis [31], further alignments were constructed using Clustal Omega [32], and viewed using Jalview [33]. BRIG was used to generate circular comparisons of genomes and Easyfig was used to create BLAST comparisons of phage genomes [34, 35].

Abriicate was used to search for antimicrobial resistance and virulence genes, and plasmid replicons using the incorporated databases ARG-ANNOT, CARD, Resfinder, VFDB and Plasmid-Finder [36–41]. A custom database was created from the Phaster [42] website database and BLAST was used to search for intact phages [43]. Potential plasmid sequences were confirmed using plasmidSPAdes and investigated using Mob-recon from Mob-suite to classify them against the database of known plasmids [44, 45].

A genome-wide association study was run using pySEER [46], using the phenotypes of 'squirrel/not squirrel' on a single MLST sequence type (ST133) to reduce the problem of lineage-related effects. A linear mixed model was used on kmers and a phylogeny incorporated to control for population structure.

RESULTS

All 88 squirrels sampled on the Isle of Arran and Brownsea Island were deemed healthy on inspection; of the 137 squirrels sampled on Jersey, 122 were free of clinical signs of FED on inspection (classified as 'healthy'), 14 had clinical signs or post-mortem evidence consistent with FED and one had other, non-FED-like skin lesions. The three diseased squirrels from IOW had skin lesions consistent with FED (Table 1).

S. aureus isolates were recovered from 43 red squirrels out of 228 sampled, 26 of 210 healthy squirrels (12.4%), including 1/122 healthy squirrels from Jersey, and from 17/18 (94.4%) squirrels with skin lesions (Table 1). On Jersey, this is likely to underestimate the true carriage rate because posting delays might have resulted in false negative samples; the average time from sampling on Jersey to laboratory was 5 days with a

Table 1. Occurrence and molecular characteristics of *S. aureus* isolated from carriage or surface infection sites of 228 red squirrels, 20 sheep and 10 red deer at four locations: The Isle of Arran, Brownsea Island, Jersey (Channel Islands) and IOW. The percentage positive for *S. aureus* for IOW is not reported as only suspected FED cases were sampled. MLST of isolates was deduced from whole-genome sequences. The allele sequences are ST133 (6,66,46,2,7,50,18), ST2328 (251,66,46,2,7,50,2), ST130 (6,57,45,2,7,58,52), ST49 (14,16,11,2,13,12,14), ST1957 (216,16,11,2,13,12,14), ST692 (12,89,1,1,4,5,90), ST188 (3,1,1,8,1,1,1), ST8 (3,3,1,1,4,4,3), ST1958 (3,3,1,1,4,226,3), ST425 (18,33,6,20,7,50,48), ST1640 (6,125,213,27,13,13,15), ST3237 (6,380,6,18,62,70,406). ~Individuals co-colonized with two different STs. * Individual coinfecting with ST49. \$ Individual not confirmed as FED.

Location		Animals sampled	Positive for <i>S. aureus</i> (%)	MLST (allelic patterns in legend and Table S3)	Individuals carrying each sequence type	Isolates in each sequence type
Arran	Squirrel	44	14 (32)	ST133	7	15
				ST2328	6	18
				ST49	1	3
	Sheep	20	13 (65)	ST1640	10	21
				ST130	2	4
				ST8	1	2
	Red Deer	10	8 (80)	ST3237	8	15
				ST1640	2~	2
				ST130	1~	3
				ST1958	1~	1
				ST425	1~	1
Brownsea	Squirrel	44	11 (25)	ST692	4	7
				ST133	4	9
				ST188	2	5
				ST130	1	3
Jersey	Squirrel	137	16 (12) (FED <i>n</i> =14, not FED <i>n</i> =2)	ST49	14 (FED)	88
				ST2328* \$	2 (FED/not FED)	3
				ST130	1 (healthy)	8
IOW	Squirrel	3	2 (FED)	ST49	1	16
				ST1957	1	4

maximum of 41 days. *S. aureus* was also isolated from 13/20 (65%) sheep and from 8/10 deer (80%) (Table 1).

Characterization of the *S. aureus* isolates

Genomes

A total of 228 genomes was sequenced using Illumina sequencing and genomes were assembled with a mean of 63 contigs (Table S1). Assemblies with N50 lower than 10000 (*n*=4) were removed from core gene alignments constructed from the pangenome as the poor assemblies contained many apparent gene alleles, which distorted the branch lengths in the phylogenies.

In order to provide closed, accurate, closely related assemblies for mapping, and to enable examination of the context of mobile genetic elements, two red squirrel isolates from Jersey (ST49) and one from the Isle of Arran (ST133) were additionally sequenced on the Oxford Nanopore platform. Two ST49 isolates were chosen because they had different bacteriophage content, including the presence or absence of the phage carrying *lukM/F* genes (henceforth referred to as 'the *lukM* phage'). Using a combination of long- and short-read data, complete assemblies consisting of a single closed chromosome with no plasmids were generated for all three isolates (Table S1). Genome data is publicly available at <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA679188>

S. aureus lineages recovered

The *S. aureus* lineages recovered are reported in Table 1.

Of the 44 healthy red squirrels from the Isle of Arran that were sampled, *S. aureus* was isolated from 14 of them. Three lineages of *S. aureus* were observed; seven individuals carried ST133, six individuals carried ST2328 and a single individual carried ST49. Healthy red squirrels on Brownsea Island (11/44) were found to carry four different lineages; four individuals carried ST692, four individuals carried ST133, two individuals carried ST188 and a single individual carried ST130.

Healthy sheep (*n*=13/20) from a single flock on the Isle of Arran, which grazed two sites close to the red squirrel sampling site carried ST1640, ST130 and ST8. Healthy free-ranging red deer from Arran (*n*=8/10) carried ST3237, 1640, 130, 1958 and 425. The deer were frequently (4/8) coinfecting with more than one sequence type.

On Jersey, 14 squirrels, which had clinical signs or post-mortem evidence consistent with FED were found to carry ST49, and one of these was co-infected with ST2328. One squirrel, which had skin lesions not confirmed to be FED, also carried ST2328. A single healthy squirrel carried ST130.

On the IOW, of three red squirrels with skin lesions, one carried ST49 and one carried the single-locus variant of ST49,

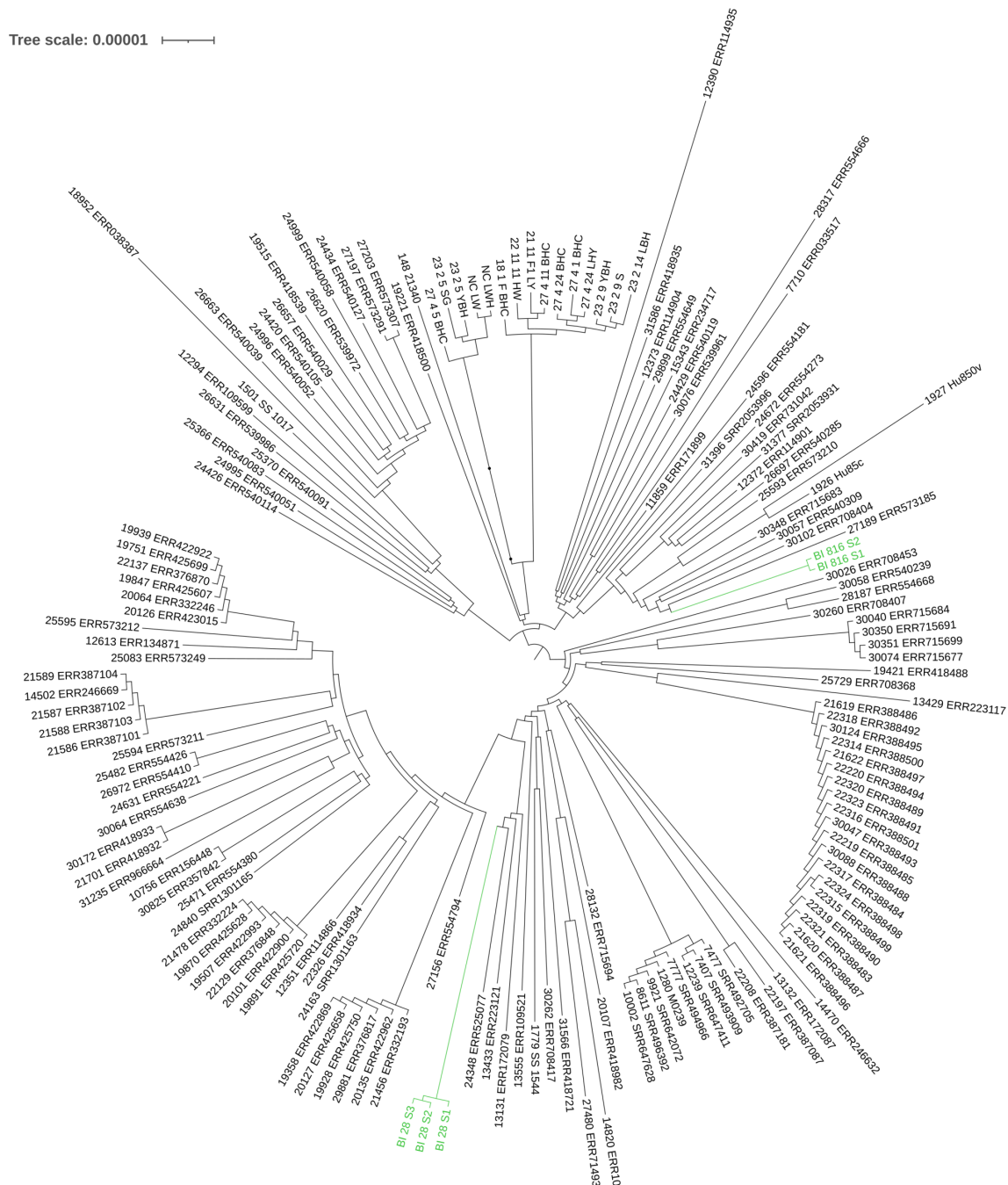


Fig. 1. Phylogeny constructed using a core-gene alignment from Roary using publicly available genomes of ST188 and genomes from red squirrels (Brownsea – green). The genomes from squirrels appear to represent two separate acquisitions. Available on Microreact https://microreact.org/project/_WmBHj3Ru.

ST1957, which was also reported by Simpson *et al.* (2013) on this island [6].

The lineages recovered from red squirrels have previously been recorded in other host species: ST133 and 2328 (ruminants), ST130 (small ruminants and small mammals), ST692

(avian), ST188 (livestock and human), ST49 (small mammals and pigs), ST1957 (red squirrels).

Phylogenomic analysis suggests multiple host switch or spillover events

Genome sequences corresponding to sequence types ST188, ST133 and ST49 were compared with publicly available

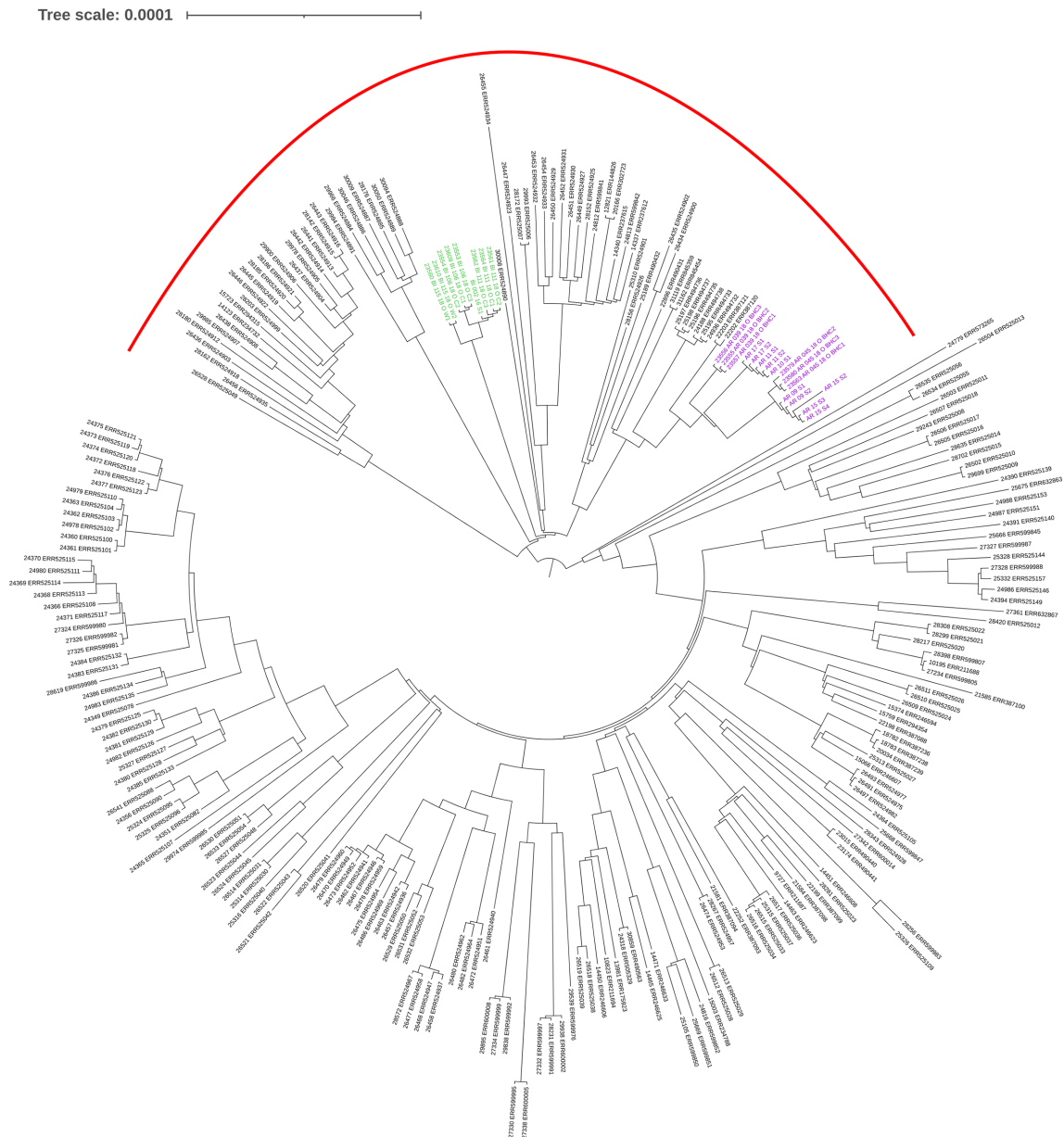


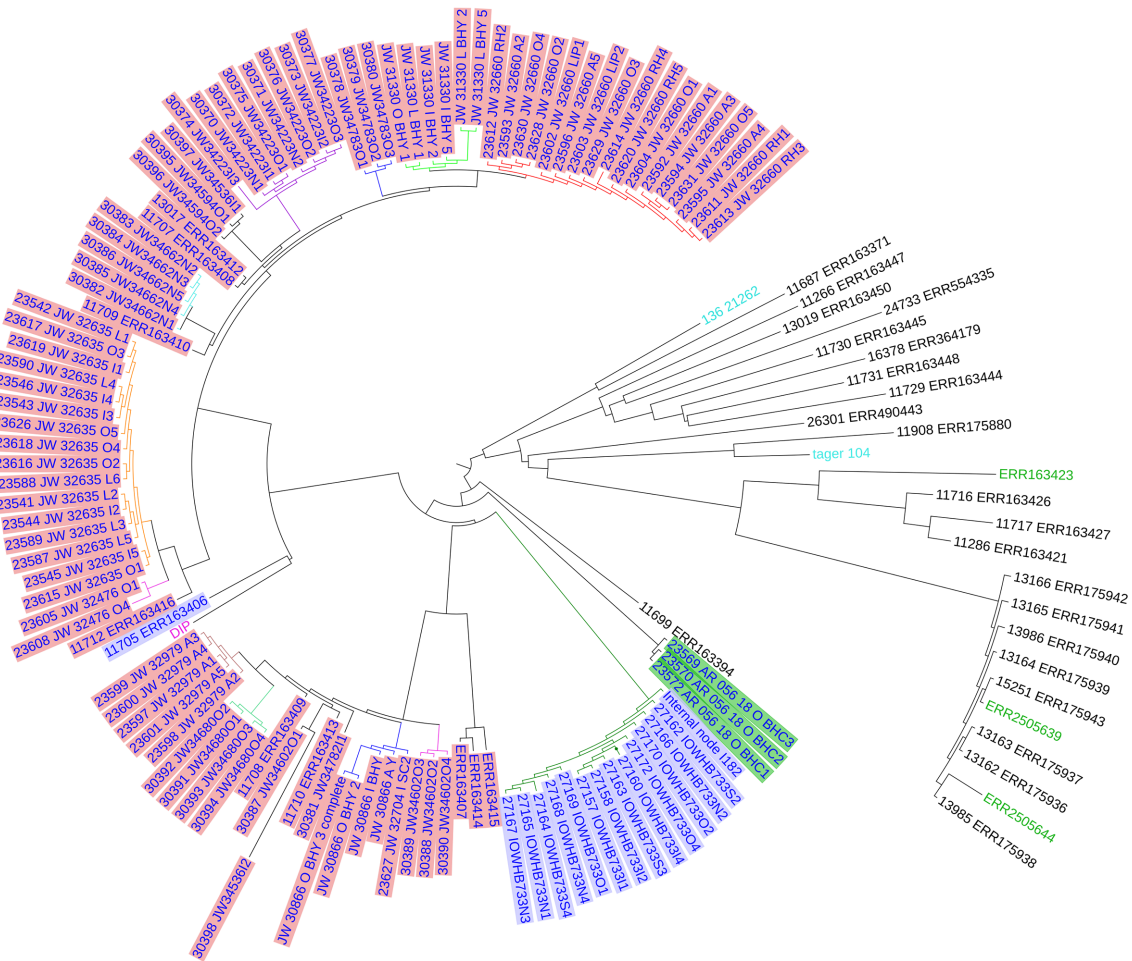
Fig. 2. Phylogeny constructed using a core-gene alignment from Roary of publicly available genomes of *S. aureus* ST133 and genomes of isolates from red squirrels on the Isle of Arran (mauve) and Brownsea Island (green). Red bar marks the clade with isolates which do not contain the lukM phage. This tree is available on Microreact: <https://microreact.org/project/n4biBCzju>.

genomes for these lineages by constructing phylogenies using core gene alignments generated by pangenome analysis using Roary (Figs 1–3). The remaining sequence types did not have enough publicly available genomes to construct useful phylogenies.

On the ST188 tree, five ST188 strains isolated from two squirrels on Brownsea Island were resolved into two distinct sub-clusters corresponding to the individual squirrels from which they were isolated. They are positioned far apart on the tree (Fig. 1), suggesting independent introductions into the squirrel population. Although neither cluster is closely

related to any of the publicly available genomes for this lineage, it is possible to infer that these isolates may have been acquired from humans based on phage content. All five squirrel isolates carried the β -haemolysin converting phage (*Saphi3*), which harbours genes associated with evasion of the human immune system such as *sak*, *chp* and *scn*, commonly termed the immune evasion complex (IEC). This phage has been reported to be more common in isolates recovered from human sources and is often absent from those recovered from animal sources [47]. Additionally, isolates from the two squirrels have a different combination of IEC genes (*sak*,

Tree scale: 0.0001



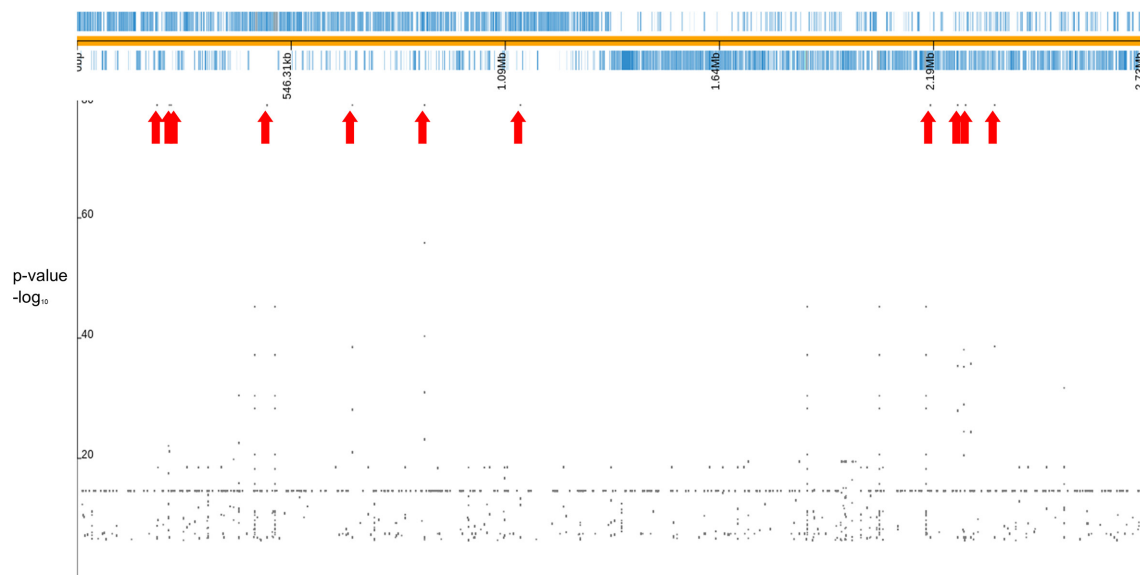


Fig. 4. Manhattan plot of significant k-mers in squirrel isolates from a linear mixed model GWAS of isolates in ST133 using pySEER, plotted against the closed genome (AR39_merged_O1) visualized using Phandango [72]. The x-axis is the genome with open reading frames (top) and the y-axis is the P -value $-\log_{10}$. Red arrows indicate most significant kmers.

we cannot completely rule out multiple transmissions from unsampled hosts, which do not appear in our phylogeny. For most of the publicly available genomes there is no information as to the host from which they were isolated, which limits the power of this analysis to identify possible reservoir hosts. To address this, we mapped those genomes for which host metadata was available against our closed ST133 assembly (from the Isle of Arran) and reconstructed a phylogeny based on genome-wide SNPs (Fig. S1). However, there is no clear correlation between phylogeny and host species, and we cannot infer the source of the isolates carried by red squirrels. However, this analysis does confirm that there are three distinct sub-clusters and greater overall diversity in the isolates from the Isle of Arran than those from Brownsea Island suggesting that these squirrels have been carrying these strains for longer.

Mobile genetic elements in CC133

Mobile genetic elements (MGE) such as phages and pathogenicity islands often carry genes that contribute to host adaptation and an examination of these elements could assist with identifying the host source of isolates found in red squirrels, and to assess the extent to which these isolates might have become adapted to squirrels. Carriage of the lukM phage has been reported to be common in the genomes of ruminant strains from multiple lineages, in which it helps *S. aureus* evade the bovine immune system and contributes to its virulence [12]. None of the ST133 isolates from squirrels contained this phage, however neither do other isolates in the same clade in the phylogeny (Fig. 2 marked with red bar) [13]. Phages and pathogenicity islands of ovine strains have previously been characterized primarily using the closed genome ED133 as an ST133 reference genome [49], and this and other

sheep-derived isolates have been reported to carry specific ovine phages (including a lukM phage) and pathogenicity islands. We examined squirrel isolates in ST133 and ST2328 for the presence or absence of these elements and found them to be absent except in ST2328 isolates from the Isle of Arran (Table 2). However, as with the lukM phage we also found that other isolates within the clade lack these elements, it is therefore not clear if the isolates lost them before or after transmission to squirrels from another host (Fig. 2).

Other phages

Further examination of phages in isolates from red squirrels in ST133 and Arran ST2328 found that there is an intact phage inserting into the *hly* gene (Table 2), but the phages from each lineage only share around 30% BLAST similarity with each other (Fig. S2). The *hly* gene is the usual site of integration of the *S. aureus* phage 3, which carries the human IEC genes, and phage insertion will result in loss of function of this gene, but the *hly*-converting phages in the red squirrel isolates do not carry any IEC-like genes in the expected site next to the amidase/holin module. ED133 does not carry this or any other phage at this site, and neither do the Jersey ST2328 isolates.

Genome wide association study in ST133

When bacteria switch to a new host from a host species to which they are adapted, a period of rapid adaptation may ensue. Previous studies have described an initial loss of genetic material following host switching, particularly of MGEs, but other adaptations including SNPs, insertions or recombinations have also been described [50]. Because the phylogeny of ST133 strains suggested that there is likely to

Table 3. Top hits from a GWAS of isolates in ST133 using pySEER plotted against the completed genome AR39_merged_O1. The protein product is as predicted by Prokka and the likely functional category of the protein is inferred from the available literature on these or similar proteins. Variant type is synonymous (s) or non-synonymous (ns) SNP. AR=Isle of Arran isolates only. BI=Brownsea Island isolates only

Gene	Annotation	Predicted product	Category	Notes	Variant
AR39merged_00164	<i>coa_4</i>	Staphylocoagulase precursor	Virulence	Interaction with host prothrombin	AR nsSNP
AR39merged_00187	<i>tarK</i>	Putative polyribitolphosphotransferase		Cell wall synthesis. Immune evasion.	AR sSNP
AR39merged_00191	<i>tarL</i>	Putative polyribitolphosphotransferase		Cell wall synthesis. Immune evasion.	BI sSNP
AR39merged_00425	<i>murJ</i>	MOP superfamily PST family polysaccharide transporter	Carbohydrate metabolism	Membrane	AR sSNP
AR39merged_00634	<i>fruA</i>	PTS system D-fructose-specific transporter	Carbohydrate metabolism	Membrane	AR nsSNP
AR39merged_00809	<i>cdr</i>	Coenzyme A disulfide reductase	Metabolism		AR nsSNP
AR39merged_01042	<i>carB_1</i>	Putative carbamoyl phosphate synthase	Amino acid metabolism		AR nsSNP
AR39merged_02040	<i>htsC</i>	Iron-binding ABC superfamily ATP-binding cassette	Iron metabolism	Transmembrane transport of staphyloferrin	AR nsSNP
AR39merged_02117	<i>fmbB_1</i>	Peptidoglycan pentaglycine interpeptide biosynthesis	Metabolism	Cell wall synthesis	AR nsSNP
AR39merged_02141	<i>utp_1</i>	Membrane-bound urea transporter	Metabolism	Membrane	AR nsSNP
AR39merged_02211		Hypothetical protein		Between <i>tca</i> (membrane-associated) and <i>hss</i> (trans-membrane haem sensing) operons.	AR nsSNP

Abbreviations; MOP; multidrug/oligosaccharidyl-lipid/polysaccharide, PST; polysaccharide transport, PTS; phosphotransferase system.

have been independent introductions of this lineage into the squirrel populations of the Isle of Arran and Brownsea Island, we carried out a GWAS to establish if there was genomic variation specific to both of these squirrel populations but absent from other ST133 genomes. We included 241 publicly available genomes, 15 squirrel isolate genomes from the Isle of Arran and nine from Brownsea Island. We used pySEER with a linear mixed model and k-mers [46]. Significant k-mers were filtered using a *P*-value of 2.80E-07 (determined from the number of unique k-mer patterns) and mapped against the closed genome in ST133 from this study (AR_39_merged_O1) (Fig. 4).

This highlighted significant hits in 11 genes spread across the length of the genome, of which six coded for cell-wall metabolism or transporter proteins, one coded for staphylocoagulase (*coa*), which interacts with host prothrombin, three coded for proteins in metabolic pathways, one was a urea transporter and one coded for a hypothetical protein, which was located between two membrane-associated operons (Table 3). This prominent representation of cell-wall protein genes may suggest host adaptation, as these proteins are likely to interact strongly with the host immune system. However, all except one of these 11 gene variants were found only in isolates from the Isle of Arran, not from Brownsea Island,

and there were no common variants detected. Moreover, the single variant associated with the Brownsea Island population is synonymous, whereas 8/10 of the Isle of Arran SNPs are non-synonymous, which is consistent with a role in adaptation. The variants reported here were present in all the isolates from the Isle of Arran (or Brownsea), and in none of the other isolates in the analysis.

ST692 strains have previously been recovered from game birds and are part of the clonal complex CC385, which is an avian-specific clade. A previous study reported a set of genes that were unique to avian strains of *S. aureus*, including those within CC385 (Table S4) [51]. The authors kindly provided the sequences for these genes and we undertook a BLAST search for them in ST692 strains isolated from four squirrels on Brownsea island and found that some, but not all of the avian-unique genes were present, suggesting that these strains were likely acquired by red squirrels from avian sources but might have subsequently undergone some adaptation to squirrels [43].

S. aureus ST49 was isolated from red squirrels on Jersey and IOW with clinical signs typical of FED. The single locus variant ST1957 was isolated from a squirrel on IOW with FED, but is not included in the phylogeny (Fig. 3). ST49 was also isolated from one apparently healthy red squirrel on the

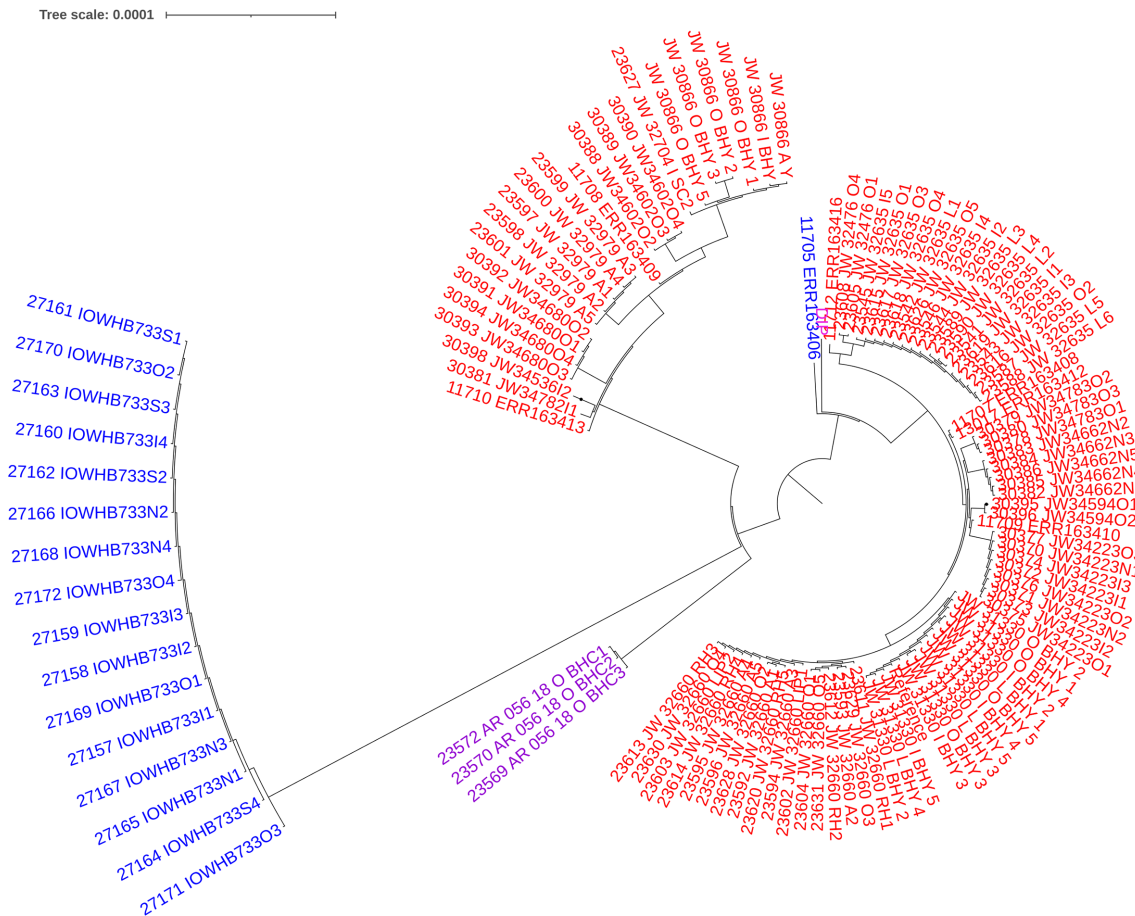


Fig. 5. Phylogeny of ST49 isolates from red squirrels from a whole-genome-mapping alignment. Leaf label colours: red=Jersey red squirrels, dark blue=IOW red squirrels, mauve=Isle of Arran red squirrels, pink=Germany bank vole. The small branch lengths of the mini-clades illustrate the low within-host diversity in this collection. This tree is available on Microreact: <https://microreact.org/project/tcJGv5uKjAmMHWBTyGPxv>

Isle of Arran which was caught and released, so we cannot be sure that it did not subsequently develop disease.

The publicly available genomes, representing isolates from humans, pigs and other unknown sources, are distinct from the squirrel isolates on the ST49 phylogeny (Fig. 3), and none of these 26 isolates contain the lukM phage. In contrast, this phage is present in all but four of the 107 squirrel isolates plus the isolate from a bank vole (DIP), and the four exceptional isolates were obtained from one swab from each of two squirrels. The genome content of the lukM phage is nearly identical (99% identity) where it is present and will be fully described elsewhere. A further distinction is that the ST49 isolates from squirrels have no plasmids whereas the publicly available reference ST49 isolates have between 1 and 4, many of which are previously described (Table S5).

A phylogeny of the clade containing the squirrel and bank vole isolates was then constructed using a whole-genome alignment of isolates mapped to the closed assembly from

a squirrel, which contained the lukM phage (JW31330_O1_dhc) (Fig. 5).

From Fig. 5 it is clear there have been limited introductions in each geographic site, and no migration between them. In order to explore transmission within Jersey between individual squirrels, we undertook a principal component analysis using SNP distances on each of the two separate clades of isolates from Jersey in the tree (Fig. S3). This showed four pairs of isolates, which clustered together, and the distances between each pair were between three and 23 SNPs (3–5, 5–6, 9–10 and 23), whereas the within-host diversity was less than three SNPs. Each of these pairs of isolates were recovered from squirrels found in similar geographical locations. Therefore, for three of these pairs it is not possible to rule out a squirrel-to-squirrel transmission, however we might also expect to see this pattern of SNP distances and topology of the phylogeny if squirrels were acquiring infection from an unsampled reservoir host in the same location.

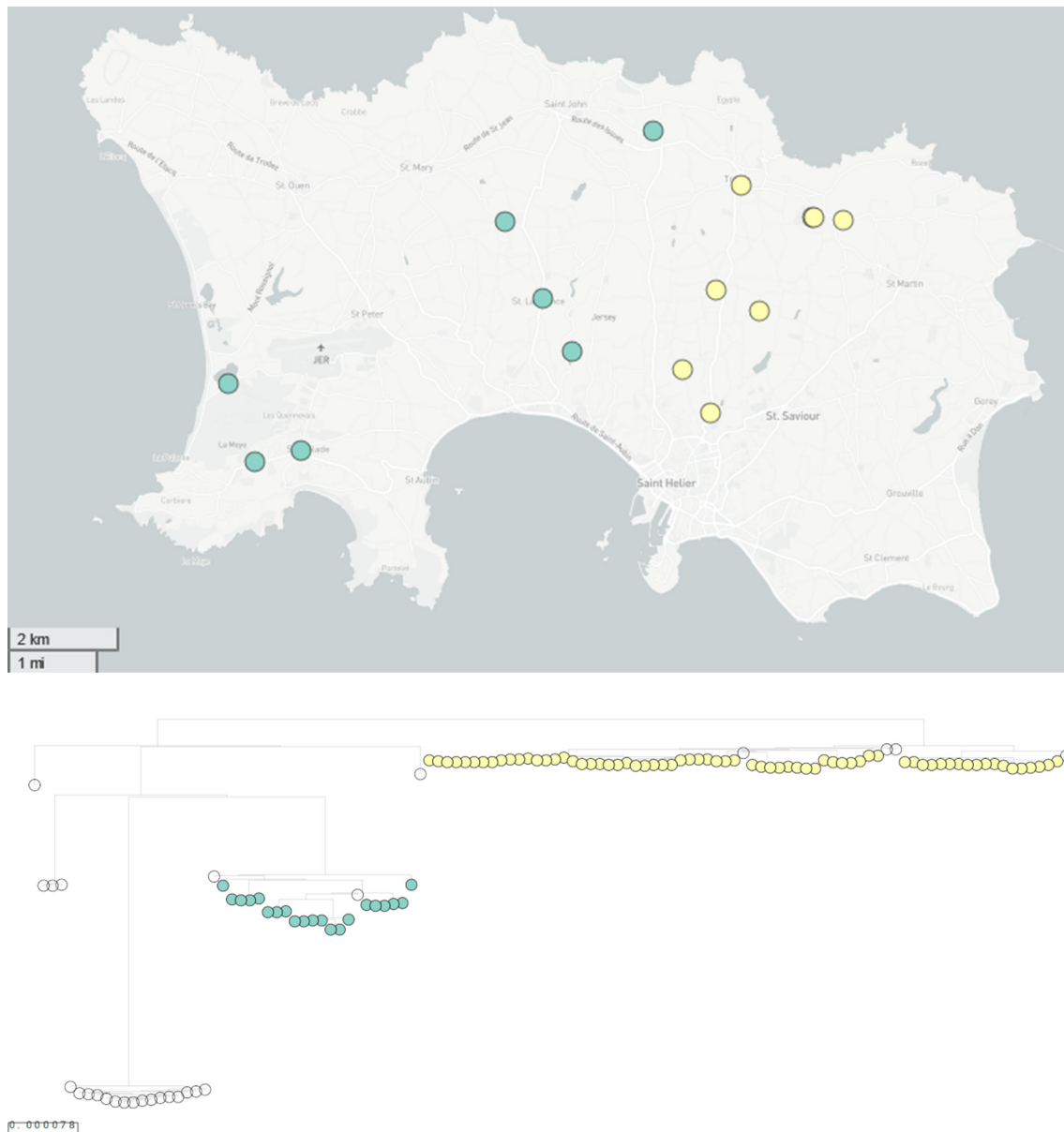


Fig. 6. Map of Jersey (Channel Islands) showing the locations red squirrels were found (top) and a phylogeny of ST49 isolates from a whole-genome-mapping alignment with the two Jersey clades coloured (bottom). Clear circles are isolates from IOW and Isle of Arran. (Same tree as Fig. 5.)

The red squirrel isolates from Jersey cluster into two separate clades, which correspond to an east-west split of the location where the squirrels were found (Fig. 6). The SNP distances between isolates in these two clades are between 250–300 SNPs, and BLAST analysis using BRIG demonstrated that the SNPs are spread along the genome (Fig. S4)

In an earlier study as part of the Jersey red squirrel surveillance project, 169 red squirrels had tissue samples taken for DNA and mitochondrial DNA sequencing revealing the existence of two mitochondrial control region haplotypes [52]. The geographical location of squirrels with these haplotypes shows a strong footprint, which is believed to

result from the introduction of squirrels from either British or European stock (Fig. 7). While the two haplotypes are not completely separated, the east-west division strongly echoes the two clades of ST49 found in red squirrels (Fig. 6). The geography of Jersey clearly presents a barrier to mixing of animals from the east and west of the island. The barriers could be man-made, for example roads, or natural, such as breaks in woodland through which animals disperse.

Within-host diversity in ST49 infected red squirrels

Multiple sampling of several sites on a single host and of several isolates from each swab allows an estimation of the

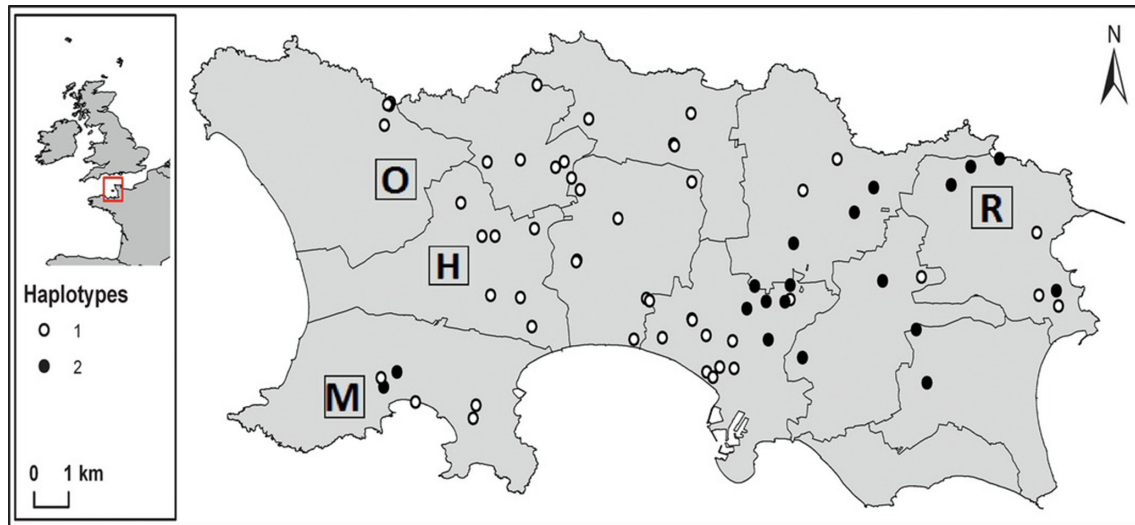


Fig. 7. Map of location of the two mtDNA haplotypes found in red squirrels on Jersey (Channel Islands). Haplotype 1 (white circles) is most similar to British haplotypes and haplotype 2 (black circles) is most similar to European haplotypes. The sites where red squirrels are documented to have been introduced are marked as follows: Rozel (r), La Hague Manor (h), La Moie House (m) and St Ouen's Manor (o). Reproduced from Simpson *et al.* (2013) under Creative Commons License 3.0 [52].

within-host diversity. A host which has carried a strain for a longer time, has been infected with a more diverse inoculum or been infected on multiple occasions, would be expected to have a greater diversity of isolates [53]. We obtained multiple ST49 isolates from 14 red squirrels, so to estimate within-host diversity a SNP distance matrix was constructed from

the mapping alignment using snp-dists and approximate duration of carriage calculated based on published *S. aureus* mutation rates (Table 4) [54]. In general, within-host diversity appears very low (maximum 2–3 SNPs), which suggests that ST49 is typically not carried for more than 2 months within each squirrel. This is consistent with the lack of evidence of

Table 4. Number of SNPs in a core-genome alignment of ST49 multiple isolates cultured from single hosts to assess within-host diversity. Divergence time (years)=[maximum pairwise SNP distance/(mutation rate x number of sites in alignment)]/2. Number of sites in alignment is 2.726 Mbp. A mutation rate of 2.7 SNPs per MB per year is used to estimate the length of time carried [54].

ID	No. of isolates	Max SNP	Min SNP	Estimated length of carriage (years)	Estimated length of carriage (days)
JW_32635	16	3	0	0.20	74
AR_056_18	3	0	0	0.00	0
JW_32660	17	2	0	0.14	50
JW_32979	5	2	0	0.14	50
JW_32476	2	1	0	0.07	25
JW_30866	6	2	0	0.14	50
JW_31330	14	1	0	0.07	25
JW_34223	8	1	0	0.07	25
JW_34783	3	2	1	0.14	50
JW_34662	5	0	0	0.00	0
JW_34602	3	1	0	0.07	25
JW_34680	4	2	0	0.14	50
JW_34594	2	0	0	0.00	0
IOW_HB733	13	0	0	0.00	0

squirrel-to-squirrel transmission in the phylogeny and also with the clinical time-frame of the disease in which death occurs in days once the squirrel has become sick enough to be captured.

DISCUSSION

The carriage rate of *S. aureus* in red squirrels in this study varies according to the island location (12–32%). However, in healthy squirrels on the Isle of Arran and Brownsea Island the rate was between 25–30%, which is comparable to that reported in humans [55]. The strains carried also showed considerable variation dependent on the island and the presence of sympatric species. This tendency to acquire strains from other hosts in the environment has also been reported in wild and captive rats in Europe, and in that case the clones also included CC49 and CC130 [8]. However, the evidence in red squirrels here points to both recent spillover events, and possible host switches involving a longer time scale and some genomic adaptation to the new host [50].

Healthy red squirrels on the Isle of Arran (14/44) were found to carry STs 133, 2328 and 49 (Table 1). ST133 is commonly isolated from small ruminants, especially sheep, but also other hosts including cattle and wildlife [56, 57]. ST2328 is a double-locus variant of ST133, which is a rarely reported isolate of sheep [17]. It is noteworthy that in our limited sampling of sheep and deer close to the red squirrel sampling site on the Isle of Arran we did not recover any ST133 isolates, which may point to a host switch occurring prior to the red squirrels' introduction to the island, anecdotally reported in the 1930s and 1950s (P. Lurz, personal communication). The carriage rates of *S. aureus* in sheep (65%) and red deer (80%) appear to be high, but when the small sample sizes are accounted for, the rate in sheep is broadly consistent with reported rates of 30–45%, and a study in free-ranging red deer in Lombardy (Italy) also reported a nasal carriage rate of 91% [58][59].

Healthy red squirrels on Brownsea Island (11/44) were found to carry STs 692, 133, 188 and 130. ST692 has been isolated from farmed game birds in the UK, and poultry meat in South Korea, and is part of the avian specific clade CC385 [60, 61]. ST188 is geographically widespread and found in many different host species, including humans [62]. ST130 is also predominantly associated with ruminants, although it is also found in humans, and was the main lineage found in hedgehogs in Sweden and rats in central Europe [63, 64, 8].

On Jersey, despite sampling over 100 healthy red squirrels, ST49 was not recovered as a normal carriage isolate. However, in contrast with other islands, no other *S. aureus* carriage isolates were recovered, apart from one squirrel carrying ST130 and two with skin lesions carrying ST2328, which means that around 88% of squirrels were not colonized. Therefore, another explanation for the failure to find ST49 in red squirrels on other islands might be the possibly protective effect of carriage of less virulent strains such as ST133. This could be mediated through general immunity to *S. aureus* stimulated by the less virulent strains, or by direct inter-strain

competition such as has been observed between *S. epidermidis* and *S. aureus* in humans [65].

On the Isle of Wight two out of three red squirrels with clinical signs of FED carried *S. aureus*, one ST49 and a second one the single locus variant ST1957, which was also reported in the 2013 study [6]. The significance of this apparent greater diversity of strains causing disease on IOW is unclear and merits further study.

Red squirrels on Brownsea Island are in close contact with other wild and farmed species, and are regularly fed by humans. Avian strains (ST692) found here are consistent with acquisition from feeders known to be shared with farmed game birds. The ST188 isolates recovered were possibly acquired from humans because strains isolated from humans usually contain a β -haemolysin converting phage carrying IEC genes, while similar strains isolated from animals do not [47, 54, 66]. However, the time scale of loss or gain of the phage is unclear, as experimentally it appears to be quite stable. A study transferring a human strain to gnotobiotic piglets found that while other genes were lost within hours of transfer, the β -haemolysin converting phage was still present after 16 h [67], and in a separate experiment, which passaged human strains in sheep it was still present after more than a year [68] (R. Fitzgerald, unpublished information). Hence we cannot be sure if ST188 strains were acquired recently, or even directly from humans.

The predominant strains carried by healthy red squirrels in this study (72%) are typically associated with small ruminants (ST133, 2328 and 130)[48, 49]. This observation may reflect squirrels' utilization of ruminant-grazed fields adjacent to woodland to bury caches of nuts during autumn. Alternatively, ruminant genome adaptations may also be conducive to the survival of *S. aureus* in red squirrels. Experimental evidence suggests that immediately following a host-switch, variation occurs in the MGE with loss or gain of phages and plasmids, however longer-term adaptation may involve fixation of mutations in the core genome [51, 67, 69, 68]. In a study of *S. aureus* strains infecting rabbits, a single non-synonymous SNP in the *dltB* gene appears to confer high infectivity for rabbits. The *dltB* gene encodes a cell-membrane-associated protein and alleles of this gene were found in other rabbit-infecting strains suggesting convergent evolution [69]. The GWAS on ST133 isolates carried by red squirrels in this study also highlighted variants in several genes encoding for cell-membrane-associated proteins, which suggests a degree of host-adaptation in the strains we sampled, however it is not clear if this is a permanent host-switch, and we did not find the same variants in isolates from both the Isle of Arran and Brownsea Island. GWAS analysis in *S. aureus* is hampered by strong lineage effects, and in previous studies a false positive rate of up to 70% was identified, so laboratory investigation to functionally verify the effect of the variants is needed [70].

The ST133 isolates recovered from red squirrels may have been acquired directly from small ruminants, or from an

unsampled secondary host. They cluster in the phylogeny with other isolates which have lost ovine-specific elements including the lukM phage. In contrast, in the phylogeny of ST49 isolates, those from squirrels all contain the phage, as does the isolate from a bank vole, which clusters closely (max SNP distance= \sim 500) (Fig. 3), perhaps suggesting a role for the lukM phage in virulence in squirrels and in adaptation to bank voles. In a study of *S. aureus* in small mammals in Europe, ST49 was found in bank voles (*Myodes glareolus*) and yellow-necked mice (*Apodemus flavicollis*), however while the latter carried other strains, the bank vole carried ST49 exclusively [7].

There is considerable interest in methods to identify bacterial transmission using genome sequence data [53]. Methods that utilize SNP thresholds can be misleading if within-host diversity is not taken into account, however core SNP thresholds between 23 and 40 have been suggested, with pairs of isolates under the threshold regarded as transmission events [53]. For our ST49 data neither the topology of the phylogeny nor a principal component analysis using SNP distances was able to unequivocally demonstrate whether squirrels acquired infection from other squirrels or from an unsampled reservoir host in the same location.

Ongoing disease surveillance of red squirrels in Scotland has not recorded any cases of FED. Although bank voles live in woodland and are common throughout the UK, including IOW and on Jersey, which has its own subspecies [71], on the Isle of Arran they have been found only intermittently and in small numbers, and not at all on Brownsea Island (Dorset Wildlife Trust; personal communication) [71]. This pattern precisely mirrors our findings of ST49 in red squirrels, and supports the hypothesis that the bank vole is a reservoir host for ST49 infection in red squirrels, in which it is rapidly fatal. However, an unsampled host such as rats, which have been recorded carrying CC49 strains and are numerous on Jersey, is a plausible alternative [8]. Carriage sampling of small mammals at all locations is warranted to explore the significance of all these findings.

CONCLUSION

Red squirrels carry *S. aureus* strains usually associated with other species, primarily small ruminants, possibly as a result of an historic host-switch, however where squirrels are in close contact with birds or humans they will acquire strains from these species. We speculate that on Jersey and IOW red squirrels acquire *S. aureus* ST49 strains from a reservoir species, possibly the bank vole or rat, and rapidly develop fatal disease. There is little evidence of transmission of ST49 strains from squirrel to squirrel, and it was only recovered once from squirrels on other islands. The identification of the reservoir species on Jersey is a research priority for the development of mitigation measures to reduce the incidence of fatal exudative dermatitis in red squirrels on the island. The role of lukM/F genes in virulence in red squirrels and in adaptation to other small mammals remains to be elucidated.

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Author contributions

Conceptualization: D.H.L., A.M., A.L., K.F. and E.J.F. Data curation: K.F., M.J.G. Formal analysis: K.F., M.J.G. Investigation: K.F., A.S., A.L., H.B., T.B., M.J.G. Methodology: K.F., A.L., E.J.F. Project administration: K.F., A.L., E.J.F. Resources: K.F., A.S., A.L., T.B., H.B., E.J.F. Software: K.F., M.J.G. Supervision: A.L., A.M., E.J.F. Visualization: K.F. Writing – original draft: K.F., A.L., E.J.F. Writing – review and editing: K.F., T.B., H.B., A.S., A.M., M.J.G., D.H.L., A.L., E.J.F.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

The study was approved by the Royal Veterinary College Clinical Research Ethical Review Board (CRERB URN M2016 0093) and by the University of Bath Animal Welfare and Ethical Review Body. The Jersey Society for the Protection of Animals permitted the sampling of animals under their care and the Jersey States Vet permitted the export of swab samples to the UK. Red squirrels on Isle of Arran and Brownsea Island were captured and sampled under home office licence (PPL 70/9023), Natural England License 2016–24517-SCI-SCI and Scottish Natural Heritage Licence 90896 with ethical approval from the University of Edinburgh's Animal Welfare and Ethical Review Body. The Forestry Commission Scotland gave permission to sample culled deer.

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